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(54) Title: PROCESSES FOR IMPROVED PRESENTATION OF ANTIGENS BY DENDRITIC CELLS (57) Abstract <p>The invention relates to a process for preparing dendritic cells which present complexes of peptides and MHC molecules on their surface, by contacting the dendritic cells with bacteria that present a protein on their surfaces or in the cytosol, which include the sequences of the peptides. The bacteria are preferably transformed so that they produce a heterologous protein which is expressed on their surface.</p>		

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PROCESSES FOR IMPROVED PRESENTATION OF ANTIGENS BY DENDRITIC CELLS

FIELD OF THE INVENTION

This invention relates to the presentation of molecules so that an immune response
5 can be generated, augmented, and/or improved. More particularly, it relates to the use of
dendritic cells, and bacteria which present relevant materials on their surface, and are internalized
by dendritic cells, which then process bacterial surface molecules to suitable antigens.

BACKGROUND AND PRIOR ART

The process by which the mammalian immune system recognizes and reacts to
10 foreign or alien materials is a complex one. An important facet of the system is the T cell
response. This response requires that T cells recognize and interact with complexes of cell
surface molecules, referred to as human leukocyte antigens ("HLAs"), or major
histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger
molecules which are processed by the cells which also present the HLA/MHC molecule. See in
15 this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially
chapters 6-10. The interaction of T cell and complexes of HLA/peptide is restricted, requiring
a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T
cell is not present, there is no T cell response even if its partner complex is present. Similarly,
there is no response if the specific complex is absent, but the T cell is present. This mechanism
20 is involved in the immune system's response to foreign materials, in autoimmune pathologies,
and in responses to cellular abnormalities. Much work has focused on the mechanisms by which
proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, Science 257:

880 (1992); Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Latron et al., Science 257: 964 (1992).

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs."

Dendritic cells, or "DCs" hereafter, are antigen presenting cells which are crucial in the generation of primary immune responses. See, e.g., Steinman, Ann. Rev. Immunol 9:271-296 (1991). While they are located in most tissues, they are particularly distributed in those tissues which interface with the environment (such as the skin and mucosal surfaces), and in lymphoid organs, where they serve as "sentinels" for incoming pathogens. See, e.g., Austyn, J. Exp. Med. 183:1287-1292 (1996).

DCs function, essentially, by capturing and processing antigens, and to "alert" the immune system thereby. Inflammatory signals and stimuli by infectious agents cause migration of DCs from peripheral tissues to secondary lymphoid organs. Upon activation, DCs produce various chemokines which recruit various cellular components of the immune system, their MHC Class II molecules and costimulatory molecules become upregulated, and the DCs mature. Information on DCs generally can be found in, e.g. Thomson, et al, Dendritic Cells (Academic Press, London) (1998).

In view of their role in the activation of the immune system, DCs have been studied extensively. It is known, for example, that if DCs are pulsed with peptides, or with viral particles, they can trigger both CD4⁺ and CD8⁺ T cell responses in vivo. See, e.g., Paglia, et al., J. Exp. Med. 178:1893-1901 (1993); Porgador, et al., J. Exp. Med. 182:255-260 (1995);
5 Celluzzi, et al., J. Exp. Med. 183:283-287 (1996); Zitrogel, et al., J. Exp. Med. 183:87-97 (1993); Bender, et al., J. Exp. Med. 182:1663-1671 (1995); Bohm, et al., J. Immunol 155:3313-3321 (1995); and Bachmann, et al., Eur. J. Immunol 26:2595-2600 (1996).

It is well known that bacteria are potential inducers of DC activation. See, e.g., Henderson, et al., J. Immunol 159:635-43 (1997); Rescigno, et al., Proc. Natl. Acad. Sci. USA
10 95:5229-5234 (1998) (the disclosure of which is incorporated by reference and constitutes the parent of the present application), and Rescigno, et al., in Thomson, et al., eds, Dendritic Cells, Academic Press, London (1998), pp. 403-419. It is also well known that, while cloned DCs can process exogenous viral particles to peptides which form complexes with MHC Class I molecules (Bachmann, et al., supra), soluble proteins are very poorly presented.

15 **BRIEF DESCRIPTION OF THE FIGURES**

Figures 1A-1G show FACS profiles of various surface markers after dendritic cells were incubated with either S. gordonii or latex beads. Filled histograms represent dendritic cells following treatment, open histograms are dendritic cells before treatment, and the dashed histograms are isotypic controls.

20 Figures 2A and 2B depict ³H- thymidine uptake by CTLs following their exposure to dendritic cells which had been exposed to recombinant S. gordonii expressing relevant fusion proteins on

their surface (figure 2A), or either wild type S. gordonii plus soluble OVA antigen, or soluble OVA antigen only (figure 2B).

Figures 3A, 3B and 3C summarize data which show that the presentation of antigen by dendritic cells, following phagocytosis of bacteria, is "TAP" i.e., "transporter associated with antigen processing" dependent. In figures 3A & 3B recombinant (dark circles) and wild type (open circles) S. gordonii are tested, with a TAP deficient cell line (figure 3A), or homogenous, immature, growth factor dependent long term dendritic cell line D1 (figure 3B). In figure 3C, the TAP deficient line is tested with the full length, soluble OVA protein (open circles), or a peptide derived from it, referred to infra as SEQ ID NO:3 (dark circles).

Figures 4A, 4B and 4C show results obtained when CD4⁺ cells specific for an antigen were contacted to various materials. In figure 4A, the CD4⁺ cells were contacted to dendritic cells which had been exposed to recombinant bacteria (dark circles), or soluble antigen (open squares). Wild type bacteria are presented in the open circle. Figure 4A is a measurement of 3H-thymidine uptake, and figure 4B shows IFN- γ production. In figure 4C, increasing numbers of dendritic cells were pulsed with soluble antigen, recombinant bacteria, or wild type bacteria. The same symbols were used.

Figures 5A and 5B present data showing that phagocytosis is required for presentation of antigen. In these experiments, DC cells were either treated with a drug (CCD) which inhibits phagocytosis, or were untreated. Tests using treated DC cells are represented by open squares and circles, and untreated DC cells are represented by filled squares and circles. Circles are used

in tests where the DCs were contacted with recombinant bacteria, and squares represent contact with soluble antigen.

Figures 6A and 6B summarize data from experiments designed to determine the mechanism of antigen presentation. In figure 6A, DCs were incubated with recombinant bacteria (dark circles), soluble antigen (open square), wild type bacteria and soluble antigen (dark triangle), LTA plus soluble antigen (dark square), wild type bacteria alone (open circle), LTA alone (inverted, open triangle), or were untreated (open triangle). In figure 6B, DCs were treated with high doses of soluble antigen (open square), soluble antigen plus wild type bacteria (dark triangle), soluble antigen and LTA (dark square), wild type bacteria alone (open circle), LTA alone (inverted, open triangle), or were untreated (inverted triangle).

Figures 7A and 7B present data regarding endocytic and phagocytic activities of DCs, when incubated with S. gordonii or LTA. Open squares represent DCs with medium alone, open circles represent incubation with 50 bacteria per DC, and the open triangle represents the use of 10 μ g/ml of LTA (7A). Figure 7B shows results obtained when the treated DCs were incubated with 2 μ m, FITC conjugated latex beads.

Figure 8 shows results obtained using non-pathogenic *S. typhimurium* strain Aro A (open squares), and two recombinant forms of *S. typhimurium*, designed to present complexes of MHC- Class I and antigenic peptide on their surfaces (filled in squares and diamonds).

5 The invention, which is elaborated on in the disclosure which follows, shows that the processing and presentation of antigens by DCs are improved dramatically if the desired antigen is part of a molecule presented on the surface of a bacterial cell. Various types of bacteria can be used to accomplish the goal of antigen presentation on dendritic cells. How this is accomplished is elaborated in the disclosure which follows.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

10 **EXAMPLE 1**

These experiments were designed to study how dendritic cells internalize bacteria.

In this experiment, and in the experiments which follow, cell line "D1" is used. D1 is a homogenous, immature growth factor dependent long term dendritic cell line. It is described, e.g., by Winzler, et al., J. Exp. Med. 185:317-328 (1997), incorporated by reference.

15 One of average skill could establish equivalent lines using standard techniques. Hence, the experiments which follow, while using D1, could be carried out using other cells.

The DCs are grown in culture medium (IMDM), containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine, and 50 μ M 2-mercaptoethanol, together with 30% supernatant from R1 medium, described by Winzler et al.,

20 supra. This is what is referred to whenever "medium" is referred to infra, unless there is an indication to the contrary.

DCs were incubated (5×10^5 cells/ml of medium) for periods of time ranging between 30 minutes and 18 hours, with either live, or heat killed S. gordonii, at a ratio of 10 bacteria per DC. Following the incubation periods, DCs were sampled (5×10^5 cells/sample), pelleted via centrifugation (1200 rpm for 5 minutes), followed by two washes in cold, phosphate buffered saline, and fixing in cold, 2.5% glutaraldehyde for 4 hours at 4°C. Specimens were then prepared for electron microscopy, following standard protocols. See, e.g., Glavert, Practical Methods In Electron Microscopy, 1975, pp. 33-40 (North-Holland Amsterdam), which is incorporated by reference.

Electron microscopy showed that the S. gordonii bacteria were internalized by DC via conventional phagocytosis. Bacteria were observed to be contacting the cell membrane of the DC after only 30 minutes, which induced local thickening of the plasma membrane. Other bacteria had already been internalized, and at least 20 bacteria could be seen in large phagosomes.

After four hours, bacteria were observed, in partially degraded form, inside phagolysosomes.

These results were seen whether live or heat treated bacteria were used, indicating that the internalization was not due to active, bacterial mechanisms.

EXAMPLE 2

The effect of bacterial uptake on DCs was then studied. Previously, it had been shown that a homogenous, immature, growth factor dependent long term DC line, referred to as "D1" could be induced to full maturation via inflammatory stimuli, such as TNF- α or IL-1 β , as well as by bacterial products such as lipopolysaccharide ("LPS"), or lipoteichoic acid. See

Winzler, et al., J. Exp. Med. 185:317-328 (1997), incorporated by reference. These studies were continued, via analysis of the phenotypical changes in cell surface markers observed following induction of phagocytosis. Both bacteria and latex beads induce phagocytosis. As such, samples of D1 were incubated for 18 hours, either with S. gordonii, using the conditions given in
5 Example 1, or with 2 μ m latex beads (100 beads per D1 cell). Incubation conditions for the beads were the same as those used for the bacteria.

Following the incubation, the cells were analyzed, via FACS, to determine the profile of cell surface molecules involved in DC activation and maturation. The molecules analyzed included MHC Class I molecule H-2D^b, MHC Class II molecule IA/Ed, CD80/B7.1,
10 CD40, CD 54/ICAM-I, CD86/B7.2, and VLA-4. The analysis was carried out using commercially available monoclonal antibodies against the cell surface molecules, labeled with standard materials.

The results are set forth in figures 1A-1G. The left side of these figures shows FACS profiles from D1 cells stimulated by bacteria, and the right side shows the profiles
15 following stimulation with the beads. The filled histograms show measurement following stimulation, the open histograms present the values obtained prior to stimulation, and the dashed histogram an isotype control.

The figures show quite clearly that upregulation of the cell surface molecules listed supra only occurred following stimulation with bacteria. This was the case with both MHC
20 molecules, the costimulatory molecules B7.1, B7.2, and CD40, as well as the adhesion molecules VLA-4 and ICAM-1.

EXAMPLE 3

An additional set of experiments were then carried out to ascertain whether the phenotypical pattern described supra could be correlated to a cytokine production pattern.

To do this, 3×10^5 /ml D1 cells were incubated in complete medium together with
5 S. gordonii, at a ratio of 10 bacteria per D1 cell, for 18 hours. Culture supernatants were removed, and assayed for cytokine production, using standard ELISAs, and commercially available monoclonal antibodies.

As compared to controls, D1 cells stimulated with bacteria produced large amounts of TNF-alpha and 1L-6 (12 and 30ng/ml, respectively), and limited amounts of 1L-10
10 and 1L-1B (0.2ng/ml and 50 pg/ml, respectively). The levels of 1L-12 observed were below the detectable limit of 10pg/ml, which was expected, as DCs require either MHC Class II and CD 40 engagement, or T cell antigen presentation to be induced to produce this cytokine.

In order to rule out the possibility that DC maturation could have been induced through autocrine amplification of cytokine production, D1 cells were also incubated with the
15 S. gordonii bacteria together with neutralizing anti-TNF- α antibodies. (There was no need to add anti-1L-6 antibodies, as it has been shown by Winzler, et al., supra, that 1L-6 does not stimulate DC maturation.)

The results showed that maturation was only partially inhibited, suggesting that an additional mechanism is involved in the bacterial induced maturation of the cells.

EXAMPLE 4

It was shown, supra, that bacteria induce upregulation of cell surface MHC Class I and Class II molecules. Additional experiments were carried out to determine whether bacterial induced maturation of D1 had any effect on synthesis of MHC Class I and Class II molecules.

5 To do this, D1 cells were incubated with S. gordonii (10 bacteria per D1 cell) for periods of time ranging from 1 to 36 hours, in medium described supra. Cells were then labeled with 1m Ci/ml of [³⁵S] methionine/cysteine for 30 minutes, after which they were lysed. Lysates were precleared, using standard techniques, and then incubated overnight using an anti-MHC-Class I monoclonal antibody and protein G Sepharose. MHC-Class II molecules were
10 immunoprecipitated using anti I-A polyclonal antibodies and protein G Sepharose. The immunoprecipitates were analyzed on SDS-PAGE (10% for Class I, 12% for Class II), and quantified, using standard methods.

MHC-Class I synthesis was found to be induced very slowly, reaching its peak after about 18 hours, and was sustained for several hours thereafter.

15 In contrast, upregulation of MHC-Class II proceeded much more rapidly, reaching its peak after about 1 hour, followed by a gradual decrease over 20 hours.

EXAMPLE 5

Stability of MHC-Class I and Class II molecules was also studied. In these experiments, the DCs were incubated, as described supra, but with 10% fetal bovine serum being
20 added to the medium, for 18 hours, in the presence or absence of S. gordonii (10 bacteria per DC). Cells were labeled in the same way described in example 4, after which they were chased, for varying periods of time, with an excess of cold methionine, and in the absence of bacteria.

The same amount of cells was lysed as was lysed in example 4, and MHC molecules were immunoprecipitated. Class I molecules were immunoprecipitated via overnight incubation with protein G Sepharose coated with anti-Class I monoclonal antibodies, and Class II molecules were immunoprecipitated via incubation for 2 hours with protein G Sepharose coated with anti-Class II monoclonal antibodies. Analysis on SDS-PAGE was carried out as described supra.

The newly synthesized Class I molecules in immature DCs have a very short half life (about 3 hours); however, this increased about 3 fold following bacterial activation. This contrasts with a report by Cella, et al., Nature 388:782-787 (1997), which reported on human monocyte derived DCs, and noted upregulation of MHC molecule stimulus, but not stabilization of Class I molecules.

While Class I molecules showed increased stabilization following stimulus, the rate of appearance of endoglycosidase H resistant mature forms remained the same, whether or not bacteria were present, indicating that maturation rates did not change.

Newly synthesized MHC-Class II molecules in non-activated cells have a half life of about 10 hours. This was increased about 2 fold following stimulation with bacteria.

EXAMPLE 6

It is known that, while MHC-Class I molecules present peptides derived from exogenous proteins, soluble proteins are not presented well by these Class I molecules. The process by which they are presented has been shown to be TAP dependent. Particulate antigens, which enter via phagocytosis, on the other hand, are processed and presented much more efficiently. The following experiments were carried out to determine if bacteria which express foreign proteins could, in fact, serve as a stimulatory particulate antigen.

A recombinant strain of S. gordonii was prepared, using a host vector system referred to hereafter as GP1252. See Oggioni, et al., Gene 169:85-90 (1996), incorporated by reference. The recombinant strain produced ovalbumin, or "OVA" hereafter. To elaborate, a DNA sequence encoding amino acids 48-386 of OVA was prepared, using PCR primers:

5 CTAGATCTGA CAGCACCAGG ACAC

(sense SEQ ID NO:1)

and

TAAAGCTTTA GGGGAAACAC ATCTG

(antisense, SEQ ID NO:2)

10 and standard PCR techniques. (Genbank Accession No. V00383 provides the sequence of the OVA coding molecule.)

The resulting amplification products were cloned into insertion vector pSMB55 which had been cut with restriction endonucleases Hind III and Bgl II, using standard methods.

15 The resulting construct produced a fusion protein of the OVA sequence and streptococcal protein M6. The M6 protein is known as a fusion partner for surface expression of heterologous antigens in Gram-positive bacteria.

20 Surface expression of the M6-OVA fusion protein in the recombinant GP1252 strain was confirmed, via immunofluorescence, and Western blotting, using M6 and OVA specific rabbit polyclonal antibodies. See Pozzi, et al., Vaccine 12:1071-1081 (1994), incorporated by reference. This work indicated that each recombinant bacterium presented an average of 1000 molecules of OVA on its surface.

Following confirmation that the recombinant strains of S. gordonii presented exogenous protein, an antigen presentation assay was carried out to determine if DCs could

present OVA derived antigen on their surfaces. Either D1 cells or Tap 1⁻/1⁻ DCs were seeded (1x10⁴ cells/well) in flat bottomed microtiter pates. The Tap 1⁻/1⁻ DCs were obtained from Tap 1⁻/1⁻ mice. See Van Kaer, et al., Cell 71:1205-1214 (1992), incorporated by reference. The cells were purified from murine bone marrow after 14 days of culture, followed by positive selection with anti-CD11C antibodies, coupled to magnetic microbeads. (Use of these cells permits the conclusions regarding TAP dependency, set out infra.)

The plated cells were then pulsed with either wild type S. gordonii, wild type S. gordonii which had been transformed with the fusion protein expressing vector described supra, ovalbumin, or peptide SIINFELK (SEQ ID NO: 3), for reasons set forth infra. Time periods for the pulsing ranged from 2 hours to 16 hours, in the culture medium described supra, supplemented with 2mM L-glutamine and 50μM 2-mercaptoethanol. Plates were washed, twice, in medium without fetal calf serum, and were then fixed for 30 seconds on ice, with 0.05% glutaraldehyde in medium. Any unreacted glutaraldehyde was neutralized with 0.1M PBS/glycine. Plates were then washed, 3 times, with medium supplemented with 5% fetal calf serum.

Following this washing step, 5x10⁴ B3Z T cell hybridoma cells were added. These hybridomas recognize complexes of SIINFELK/K^b, as described by Karttunen, et al., Proc. Natl. Acad. Sci. USA 89:6020-6024 (1992), incorporated by reference. These cells were seeded in a final volume of 200μl of IMDM, 5% FCS, and a supplement of 50μg/ml gentamycin. The amount of IL-2 produced by the hybridomas was quantitated, using IL-2 dependent CTLs, and a ³H thymidine uptake assay.

Figures 2A and 2B show that DCs processed and presented bacterial antigens with MHC-Class I molecules very efficiently. In figures 2A & 2B, data are presented showing ³H-

thymidine uptake of the 1L-2 dependent CTLs, when admixed with DCs and recombinant bacteria, wild type bacteria plus exogenous OVA, and OVA alone. These experiments involve 16 hours of incubation of the DCs and the added material, using differing ratios of bacteria: DC (a range of from 500:1 to 0.3 to 1 was used, where the 500:1 ratio corresponds to 1 μ g/ml of OVA).

The process of antigen presentation required at least 8 hours, because cells fixed before 8 hours of incubation presented no antigen.

Note that, as compared to results obtained with soluble antigen, a 10⁶ fold increase in presentation was observed using the recombinant bacteria. The increase cannot be attributed to an adjuvant effect, because when wild type bacteria and soluble antigen were combined, and presented to the DCs, presentation was only increased by a factor of 10.

EXAMPLE 7

As noted, supra, Tap 1/1⁻ cells were also employed to permit determination of whether loading of the antigen took place via a classical, MHC-Class I loading, which is TAP dependent, or via a non-cytosolic, mostly TAP independent pathway.

This determination was carried out by incubating the D1 and Tap 1/1⁻ cells with wild type or recombinant bacteria, as described supra. Figures 3A, B & C show these results. In figures 3A and 3C, the results of studies using the TAP 1/1⁻ cells are shown. Figure 3A presents results using the wild type and recombinant bacteria, while 3C shows the results using the complete OVA protein, and SEQ ID NO: 3. Figure 3B is a comparison to 3A, using the D1 cells. These results demonstrate the TAP dependence of the process.

EXAMPLE 8

Examples 1-7, supra deal with murine DCs, and their ability to process bacteria presenting antigens on their surface. In the experiments which follow, human DCs were used. The antigen under consideration is the C-fragment of tetanus toxin (TTFC), and the work was extended to show that CD4⁺ T cells were stimulated by DCs which had processed recombinant strains of bacteria presenting antigen on their surfaces.

Human DCs were prepared using peripheral blood monocytes ("PBMCs" hereafter), from healthy individuals, in accordance with Sallusto, et al., J. Exp. Med. 179:1109 (1994). In brief, PBMCs were isolated using standard density gradient centrifugation, and multistep Percoll gradients. The light density fraction cells were recovered, cultured at 1x10⁶ cells/ml in RPMI 1640, complemented with 10% FBS, 1mM sodium pyruvate, 0.1mM non essential amino acids 2mM L-glutamine, 25mM HEPES, 100 U/ml penicillin, 100μg/ml streptomycin, and 0.05mM 2-ME at 37°C with 5% CO₂, in the presence of 200 ng/ml of recombinant human GM-CSF and 200 U/ml of recombinant human IL-4. The medium was changed after 3 days. After 6 days of culture, cells were recovered, and depleted of CD2⁺ and CD19⁺ cells with immunomagnetic beads coated with mAbs. The resulting population was a better than 97% pure CD1a⁺ CD14⁻ DC preparation.

EXAMPLE 9

A recombinant strain of S. gordonii was prepared which expressed TTFC. The protocol presented in example 6, supra, was followed, except a nucleic acid molecule encoding TTFC was used. The construct was identical to the construct described above, except the coding region employed corresponded to nucleotides 2844-4230 of the coding region for TTFC, which

encodes amino acids 855-1316 of this protein. Western blotting was carried out, using standard methods, to determine the amount of fusion protein presented on the surface of the recombinant bacteria. This was determined to be about 1000 molecules per cell, per or about 10^{-7} ng of antigen per bacterium.

5 EXAMPLE 10

Following preparation of the materials described in examples 8 and 9, DCs (2000/well) were pulsed, for 18 hours at 37°C with either the recombinant bacteria, wild type bacteria, or soluble TTFC, at varying concentrations, in the complete medium described supra. After this pulsing step, the cells were washed, examined for cell viability using trypan blue exclusion, and then co-cultured with T cells, in triplicate.

The T cells with which the DCs were cultured were CD4⁺ T cell clones specific for TTFC. They had been prepared by limiting dilution of TTFC-specific CD4⁺ cells generated from PBMCs of healthy individuals, using standard methods. See Lanzavecchia, et al, supra. Analysis showed that these cells were CD4⁺, CD8⁻, TCR α/β ⁺, TCR γ/δ ⁻, CD 28⁺, and secreted high amounts of IFN- γ and no IL-4 when activated with anti-CD3/anti-CD28 mAbs, or in Ag-specific stimulation assays. They were strictly MHC-Class II dependent.

CD4⁺ T cell clones and DCs derived from the same individual were mixed in these experiments. A total of about $2-3 \times 10^4$ T cells were added to each well, and experiments were run in triplicate.

20 Figures 4A-4C present these results. Figures 4A and 4B depict results obtained when a fixed number of DCs (2000/well) were used. These were DCs which had been incubated with increasing amounts of recombinant bacteria (dark circle), or soluble TTFC (open square),

with the amounts having been calibrated to be equal to each other. These co-cultures were continued for 3 days, after which T cell response was determined either by measuring ^3H -thymidine uptake (figure 4A), or IFN- γ release (figure 4B).

5 In figure 4C, results are presented showing data generated when graded amounts of DCs were pulsed with 5ng/ml of TTFC, recombinant bacteria, or wild type bacteria. When bacteria were used, they were combined with DC at a bacteria to DC ratio of 50 to 1, which is equivalent (with recombinant bacteria), to 5 ng/ml of TTFC.

10 These results show that specific CD4^+ T cells were much more readily activated when the same amount of Ag was administered to DCs as surface bacterial protein than as soluble antigen. They show that 10^2 - 10^3 times less antigen is necessary to obtain comparable T cell responses when DCs were pulsed with recombinant bacteria as compared to soluble TTFCs. Incubation with as few as 1 bacterium per DC (0.1 ng/ml TTFC), was sufficient to induce significant T cell proliferation. On a per cell basis, DC's exposed to recombinant bacteria were at least 10^2 times more effective than DC's pulsed with equal amounts of soluble antigen.

15 EXAMPLE 11

A set of experiments were carried out which paralleled those of example 1, supra. Immature DCs were incubated with live bacteria, at a bacteria to DC ratio of 50:1, and analyzed by transmission electron microscopy, as set forth in example 1.

20 The shortest time period tested was 2 hours, and at this point, bacteria were already found in a proportion (less than 10%) of DCs, within membrane-bound, phagosomal organelles. After 18 hours, bacteria were seen in more than 90% of DCs, in various stages of degradation, but always in vacuoles and phagosomes, never free in the cytosol. The number of

bacteria in each DC section varied, but the majority of DCs has phagocytosed 5-20 bacteria. Cell membranes were tightly or loosely opposed to bacteria in the phagosomes, and a single organelle contained multiple bacteria in many instances. The recombinant bacteria were phagocytosed equally as well as wild type bacteria. When incubation was carried out at 4°C, no bacterial uptake was observed. No bacteria were observed to be enclosed by cell membrane protrusions, or pseudopod coils, suggesting that the bacteria were taken up exclusively by conventional phagocytosis.

EXAMPLE 12

These experiments were designed to determine if phagocytosis was necessary for effective presentation of the antigen expressed by the recombinant bacteria. This was determined by pretreating samples of DC with the drug cytochalasin D, or "CCD" (10 µg/ml), for 30 minutes at 37°C, prior to contact with the recombinant bacteria. The pretreated cells were pulsed with bacteria (50 bacteria per DC, as above, for an amount of 5 ng/ml of TTFC), or with 1 µg/ml of soluble TTFC. Experiments were run, in parallel, with untreated cells. Following pulsing, the DCs were co-cultured for 3 days with 30,000 TTFC specific CD4⁺ cells, as are described supra. All experiments were run in duplicate.

The results, presented in terms of ³H thymidine uptake, are shown in figures 5A and 5B. Figure 5A shows the results using recombinant bacteria, and show that pretreatment inhibited presentation when recombinant bacteria were used, but did not inhibit presentation when soluble peptide was used, indicating that phagocytosis was required.

EXAMPLE 13

The experiments discussed in this example were designed to investigate the mechanism of presentation in greater detail. In these experiments, equal amounts of DCs were incubated with one of the following: (i) recombinant bacteria, at a ratio of 50 bacteria per DC; (ii) soluble TTFC, at 5ng/ml, which is an amount of TTFC comparable to the 50 bacteria (i); (iii) 5 wild type bacteria, plus soluble TTFC (5ng/ml); (iv) LTA, i.e., lipoteichoic acid (10 μ g/ml), plus 5ng/ml of soluble TTFC, (v) wild type bacteria alone, (vi) LTA alone, or (vii) were untreated. In a second set of experiments, the DCs were treated with (i) 100ng/ml of soluble TTFC alone, (ii) the soluble TTFC plus wild type bacteria, or a bacteria: DC ratio of 50:1, (iii) TTFC and LTA (10 μ /ml), (iv) wild type bacteria alone, (v) LTA alone, or (vi) were untreated. In each case, incubation conditions were as set forth in the prior examples. After 18 hours of culture, the DCs were co-cultured with 30,000 CD4⁺ T cells specific for TTFC, for 3 days. ³H-thymidine uptake was measured.

These results are presented in figures 6A and 6B. In figure 6A, it will be seen that the DCs treated with recombinant bacteria induced a much stronger T cell response than the response obtained following incubation with soluble antigen. Using wild type bacteria or LTA did not change these results, suggesting that there was no adjuvancy effect on the DCs in the response obtained using recombinant bacteria. At the higher dose of 100ng/ml of TTFC, however, both wild type bacteria and TTFC increased the capacity of the DCs to present antigen, indicating that S. gordonii can exert an adjuvancy effect at sufficiently high doses of antigen.

The following experiments explore the question of whether S. gordonii could stimulate DC maturation.

EXAMPLE 14

In these experiments, a fixed number of DCs were incubated for 18 hours with
5 varying amounts of either wild type S. gordonii, or the recombinant strain described supra. The cells were washed, incubated in PBS to which 2% FBS and 0.01% NaN₃ were added. Next, the cells were tested via flow cytometry analysis, with FITC conjugated mAbs against HLA-DR, CD14, CD1a, CD86, CD40, CD54, CD80, CD83, and mAbs against CD115, MHC-Class I, as well as FITC conjugated antimouse Ig F(ab')₂, anti-rat IgG, and control rat IgG. Standard FACS
10 conditions were employed.

Regardless of whether wild type or the recombinant bacteria were used, the DCs showed dramatic increases in molecules involved in antigen presentation, including both classes of MHC molecules, CD80 and CD86 (which are costimulatory molecules), and CD54. Both CD40 and CD83 were also upregulated. In contrast, expression of CD1a was consistently
15 diminished slightly, and expression of CD115, which is the M-CSF receptor, was abolished.

These effects were dose dependent, and were also seen when heat killed bacteria or LTA were used; however, they were not seen when 2 μ m latex beads were used. Note that when a dose of bacteria was used which permitted effective presentation of peptide (a 1:1 ratio with DC), no significant changes in phenotype were seen.

The maturation of DCs is associated with reduced phagocytic and endocytic activities. This was studied in the following example.

EXAMPLE 15

In these experiments, DCs were incubated with either wild type or recombinant
5 S. gordonii, or 10 $\mu\text{g/ml}$ of LTA for 18 hours, as described supra. These were then washed, resuspended in complete medium, and pulsed with either 1mg/ml Texas-red conjugated bovine serum albumin, or FITC-labeled-2 μm latex beads. The DCs were then incubated, at 37°C, or 4°C. Uptake was measured at selected time points by adding cold PBS containing 2% FBS, and 0.01% NaN_3 . The cells were washed, four times, and analyzed via FACS. Any surface binding
10 values obtained at 4°C were subtracted from the values obtained at 37°C.

These results are presented in figures 7A and 7B. Figure 7A compares controls (DCs incubated in medium), with DCs incubated with S.gordonii or LTA, and the uptake of the conjugated BSA. Figure 7B presents the results obtained when using 2 μm FITC conjugated latex beads. The data show a marked downregulation of phagocytic and endocytic activity in
15 mature DCs.

EXAMPLE 16

These experiments were carried out to determine if phenotypic maturation was associated with cytokine and chemokine production. DCs which had been treated as described in example 15 were then cultured in 6 well plates (1×10^6 cells/well), for 18-24 hours at 37°C, after which supernatants were collected, and stored at -80°C. Standard ELISAs were carried out
20 to measure IL-1 β , TNF alpha, TGF- β , IL-6, IL-12, IL-10, IL-8, RANTES, IP-10, and MIG.

Both wild type and recombinant bacteria induced dose dependent release of large amounts of TNF alpha and IL-6. The results also show that the amounts of IL-1 β and TGF- β release increased, but the increase was not as high. IL-10 release was also observed. This is an important DC regulatory factor, which maintains DCs in an immature state and inhibits antigen presenting activity. The bacteria were also observed to promote substantial amounts of IL-12. The DCs were also observed to release constitutively high amounts of IL-8 and low levels of IP-10. When incubated with bacteria, DCs increased secretion of IL-8 and IP-10, as well as began to release RANTES and MIG in a dose dependent matter.

These patterns were also observed when DCs were stimulated with LTA.

10 EXAMPLE 17

The methodology described supra in the preceding examples was applied to *Salmonella typhimurium* Aro A, a strain which is non-pathogenic to mice.

In these experiments, 10⁴ D1 cells were combined with either *S. typhimurium* Aro A, the Aro A strain transfected with a construct encoding amino acids 46-358 of ovalbumin, (the construct used was identical to that described in example 6, supra, except for the coding region), and two Aro A colonies, transfected with a construct which encoded this ovalbumin sequence, where the coding region was under the control of inducible IPTG promoter, in the form of a GST-OVA fusion protein. The use of GST in fusion protein constructs is well known. These two recombinant strains are referred to as OVA-M and OVA-R, respectively.

20 D1 cells were incubated with the bacteria, at the ratios described supra, for 3 hours in medium to which 5% bovine serum albumin had been added, but no antibiotics. Medium was then changed for fresh medium to which 50 mg/ml of gentamicin had been added, and incubation

continued for 3 hours. Following this, the D1 cells were fixed, as described *supra*. Hybridoma B3Z, which is described *supra*, was added to measure antigen specific IL-2 production.

The results are presented in figure 8, which show clearly that complexes of MHC-Class I and an OVA derived antigen were presented following contact of the D1 cells to the recombinant bacteria. In contrast, the non-pathogenic bacteria did not do so.

The foregoing examples show that dendritic cells, when confronted with bacteria which express a protein, can internalize the bacteria, process these, and present relevant antigens on their cell surfaces as parts of MHC-peptide complexes. Such complexes are important in provoking an immune response, as is well known in the art. Hence, one aspect of the invention is a process for making a dendritic cell which presents a desired complex of an MHC molecule and a peptide antigen on its surface, by contacting a dendritic cell to a bacteria which expresses a protein which comprises the relevant antigen, under conditions which favor internalization and processing of the bacteria by the dendritic cell, leading to presentation of the antigen in a complex with an MHC molecule. These proteins can be presented on surfaces of the bacteria, or can be cytoplasmic proteins.

"MHC," as used herein, refers to all major histocompatibility complex molecules, be they Class I or Class II molecules. Human leukocyte antigens, or "HLAs," are especially preferred components of the complex.

"Bacteria," as used herein, refers to any type of bacteria which can be internalized by a dendritic cell. Both Gram positive and Gram negative bacteria can be used. While *S. gordonii* was exemplified, it will be understood that any bacteria can be used, such as other types of Streptococcus, Salmonella, Pseudomonas, and so forth.

Exemplary of the bacteria which can be used in the invention are Salmonella typhimurium, Escherichia coli, Lactobacillus strains, Staphylococcus, such as S. aureus, Pneumococcus, such as unencapsulated Pneumococcus R6, Streptococcus, Lactococcus, Mycobacterium, such as M. smegmatis, Listeria, such as L. monocytogenes, and so forth. It has
5 been observed for each of these microorganisms that, upon contact of dendritic cells with these, cell surface activation markers are modified, including upregulation of B7.2 and CD40 molecules, which is indicative of maturation of the dendritic cells. These are essential molecular precedent events for subsequent presentation of antigens, and activation of both CD4⁺ and CD8⁺ cells.

10 The protein that the bacteria present may be a naturally occurring one, but preferably is not. Indeed, it is more preferable to use bacteria which have been treated such that they have been transformed with a nucleic acid molecule which encodes a protein that contains one or more antigenic sequences of interest. Examples of such sequences include sequences found in tumor rejection antigens, antigens associated with cancer generally, such as p53
15 antigens, viral antigens, antigens corresponding to bacterial proteins other than the bacteria used as the host, and so forth. These coding sequences can also correspond to synthetic proteins comprising or consisting of antigenic sequences known to be presented by the MHC molecules of a particular dendritic cell. MHC typing is a fairly well known technique and, as has been pointed out, supra, the art is familiar with many peptides presented by particular MHC or HLA
20 molecules. Hence, if one is interested in, e.g., only peptides which are presented by HLA-A2 molecules, the transforming sequence may be one which encodes only HLA-A2 presented peptides. In the alternative, a synthetic sequence which encodes peptides presented by, e.g.,

HLA-A2, HLA-B44, HLA-C16, and so forth, can be constructed. Analogously, constructs which encode only Class II presented peptides, or defined mixes of both, may also be presented.

These constructs are preferably prepared in the form of sequences which encode fusion proteins, a portion of which is a protein endogenous to the bacterial host. For example,
5 M6 protein can be used when the host bacteria is streptococcus and surface expression is desired, but the art will be familiar with other bacterial proteins both surface and cytoplasmic associated, which can be used in place of M6, depending upon the organism being used.

As was shown, supra, the dendritic cells which result from internalization of the bacteria can be used to generate an immune response which is stronger than the response which
10 would be generated when soluble antigen is used. Hence, yet another feature of this invention is a method for improved generation of an immune response, such as T cells, B cells, cytokine production, etc., by contacting a sample capable of generating an immune response, such as peripheral blood, or any B cell or T cell containing sample, to a dendritic cell which has internalized a bacteria as described supra.

15 The dendritic cells which result from the internalization, as has been shown, are characterized by MHC Class I and MHC Class II molecules which have much longer half lives and greater stability than comparable dendritic cells which have not internalized such bacteria. Hence, these dendritic cells are also a facet of this invention.

Also a part of this invention is a process for promoting maturation of dendritic
20 cells. It is well known that for DCs to perform their function as inducers of a primary immune response, they must undergo a series of changes referred to as maturation. The examples show that maturation of immature dendritic cells can be accelerated via contact with bacteria such as those described supra.

It is important to note that the key feature of the bacteria is the presentation of the relevant molecule on a cell surface. Hence, depending upon the type of DC used, the bacteria need not be viable when contacted to the dendritic cells. This is important, as non-pathogenic, non-viable bacteria may be used in the context of in vivo therapy. S. gordonii, for example, is
5 a non-pathogenic bacteria which is found in the oral cavity of humans. Other non-pathogenic bacteria will be known to the skilled artisan.

The ability of dendritic cells to present relevant antigen can be increased via incubation with, e.g., LPS, LTA, or bacteria prior to contact with soluble antigen. Indeed, this process is yet another feature of this invention, i.e., a process for improving the presentation of
10 complexes of MHC molecules and peptides by dendritic cells, by contacting the dendritic cells in a first step, with an adjuvant like material, such as bacteria or LTA, followed by contact with a soluble antigen. Other adjuvant type materials will be known to the skilled artisan.

Further aspects of the inventions will be clear to the skilled artisan and need not be set forth herein.

15 The terms and expressions which have been employed are used as terms of description and not limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

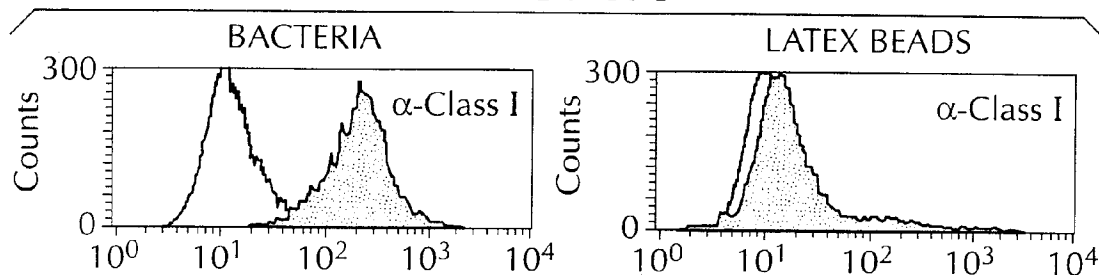
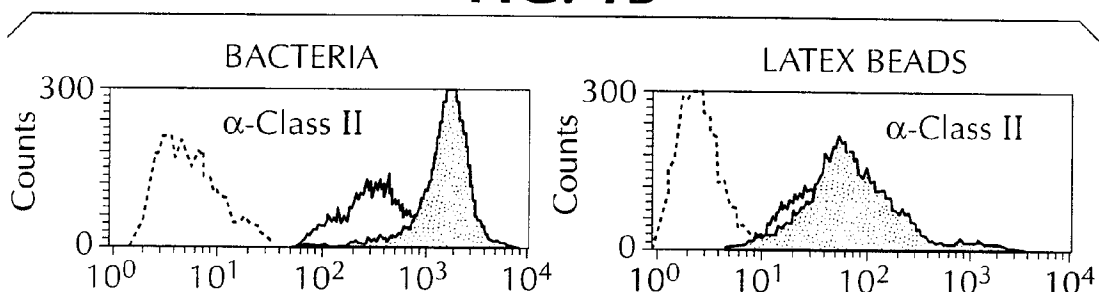
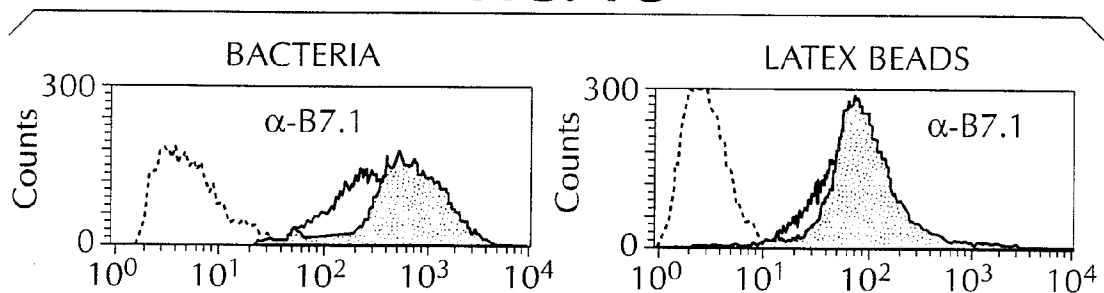
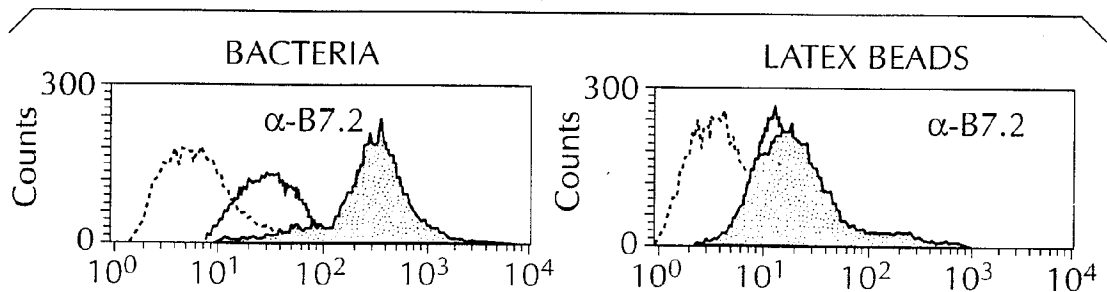
WE CLAIM:

1. A process for preparing a dendritic cell which presents a complex of an MHC molecule and a peptide on its surface, comprising contacting a dendritic cell which presents said MHC molecule on its surface with a bacterial cell which expresses a protein, the amino acid sequence of which comprises the amino acids sequence of said peptide, under
5 conditions favoring internalization of said bacterial cell by said dendritic cell, processing of said protein to said peptide, and complexing of said peptide with said MHC molecule.
2. The process of claim 1, wherein said protein is present on the surface of said bacterial cell.
3. The process of claim 1, wherein said protein is present in the cytoplasm of said bacterial cell.
4. The process of claim 1, wherein said bacterial cell has been transformed with a nucleic acid molecule which encodes said protein.
5. The process of claim 4, wherein said bacterial cell has been transformed with a nucleic acid molecule which encodes a fusion protein, a first component of which is a cell surface protein for said bacterial cell, and a second component of which is a protein which comprises said peptide.
6. The process of claim 1, wherein said peptide is presented by an MHC Class II molecule.
7. The process of claim 1, wherein said peptide is presented by an MHC Class I molecule.
8. The process of claim 1, wherein said bacterial cell is Gram positive.
9. The process of claim 8, wherein said bacterial cell is streptococcus.
10. The process of claim 1, wherein said bacterial cell is viable.
11. The process of claim 1, wherein said bacterial cell is non-proliferative.

12. An isolated nucleic acid molecule which encodes a fusion protein which comprises a bacterial cell surface or cytoplasmic protein and a protein which comprises a peptide which is presented by an MHC molecule.
13. The isolated nucleic acid molecule of claim 12, wherein said fusion protein comprises a bacterial cell surface protein.
14. The isolated nucleic acid molecule of claim 13, wherein said fusion protein comprises a bacterial cytoplasmic protein.
15. The isolated nucleic acid molecule of claim 13, wherein said bacterial cell surface protein is M6.
16. A dendritic cell which presents complexes of an MHC molecule and a peptide on its surface, prepared by contacting a dendritic cell to a bacterium which encodes a fusion protein comprising a bacterial cell surface protein or a bacterial cytoplasmic protein and a protein, the amino acid sequence of which comprises the amino acid sequence of said peptide, under conditions favoring internalization of said bacterium, processing of said protein to said peptide, and presentation of said peptide in a complex with said MHC molecule.
17. The dendritic cell of claim 16, wherein said fusion protein comprises a bacterial cell surface protein.
18. The dendritic cell of claim 16, wherein said fusion protein comprises a bacterial cytoplasmic protein.
19. The dendritic cell of claim 17, wherein said bacterial cell surface protein is M6.

20. A process for stimulating an immune response, comprising contacting a sample which contains an immunocompetent cell with the dendritic cell of claim 12, under conditions favoring an immune response to said dendritic cell by said immunocompetent cell.
21. The process of claim 20, wherein said immunocompetent cell is a T cell.
22. The process of claim 20, wherein said immunocompetent cell is a B cell.
23. The process of claim 20, wherein said immune response comprises proliferation of T cells specific for complexes of peptide and an MHC molecule on said dendritic cell.
24. A process for stimulating maturation of an immature dendritic cell, comprising first contacting said immature dendritic cell with an adjuvant like material, followed by contact with the dendritic cell of claim 16.

1/10

FIG. 1A**FIG. 1B****FIG. 1C****FIG. 1D**

2/10

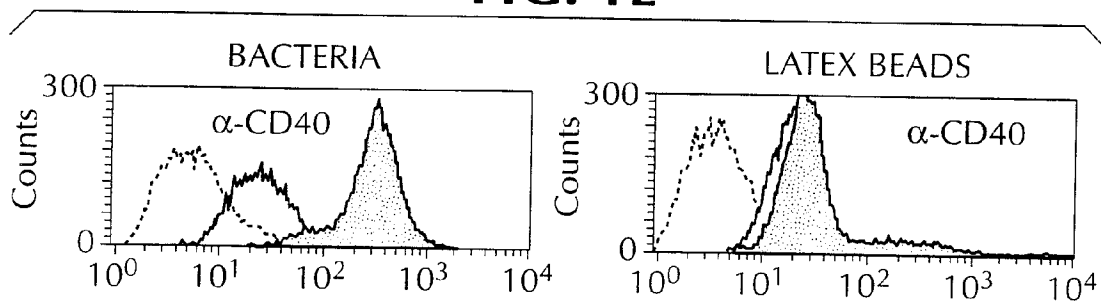
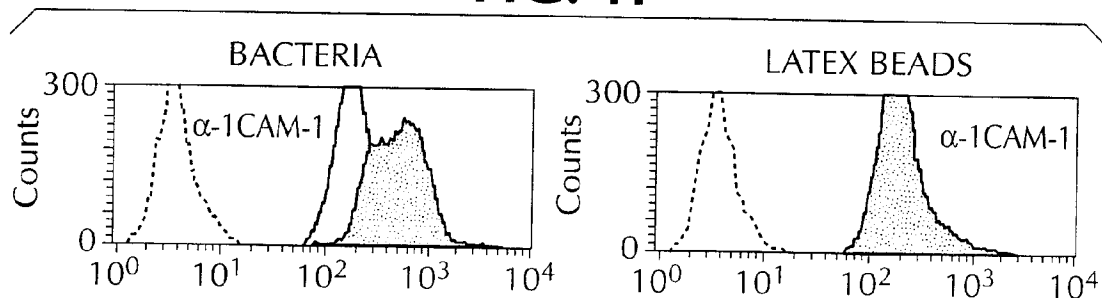
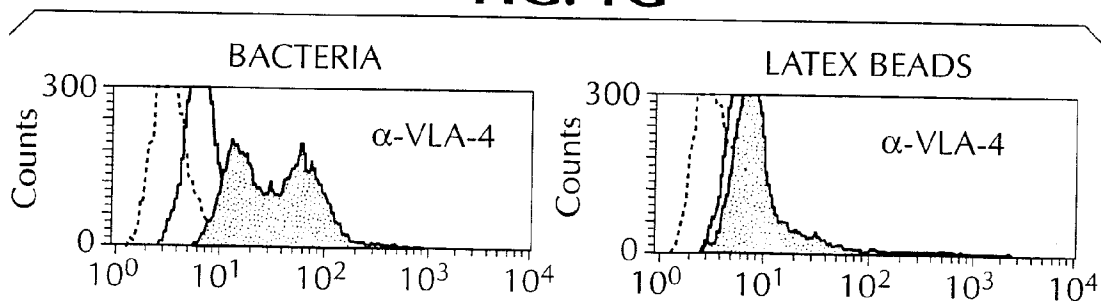
FIG. 1E**FIG. 1F****FIG. 1G**

FIG. 2B

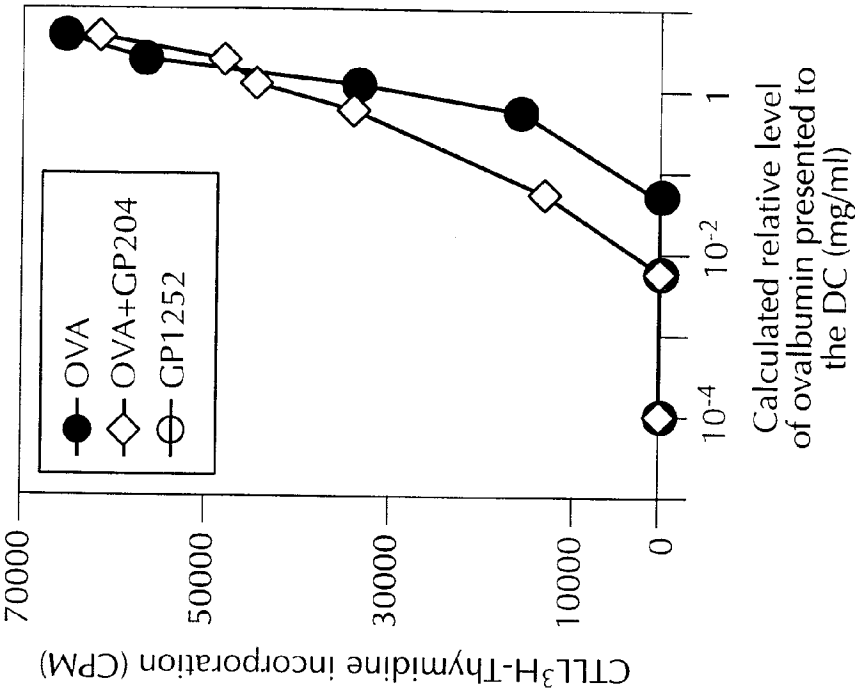
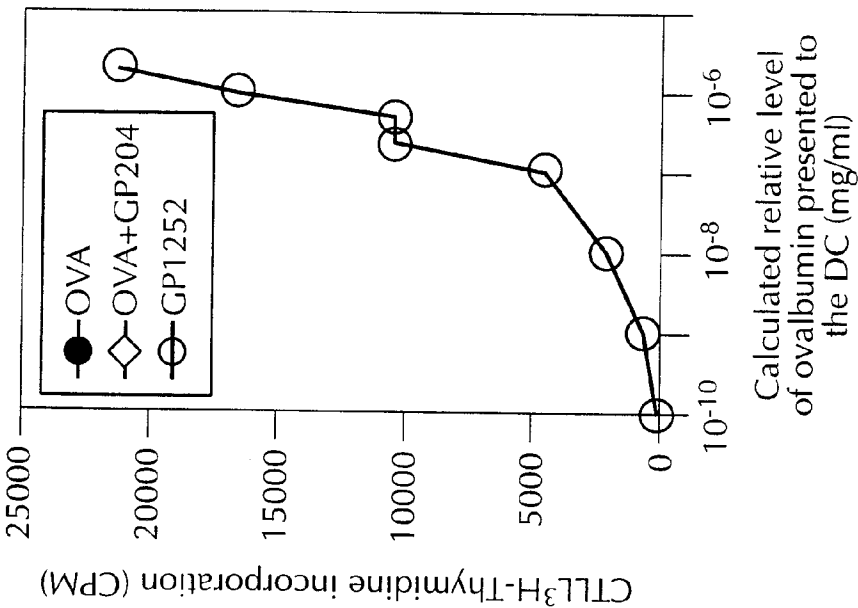
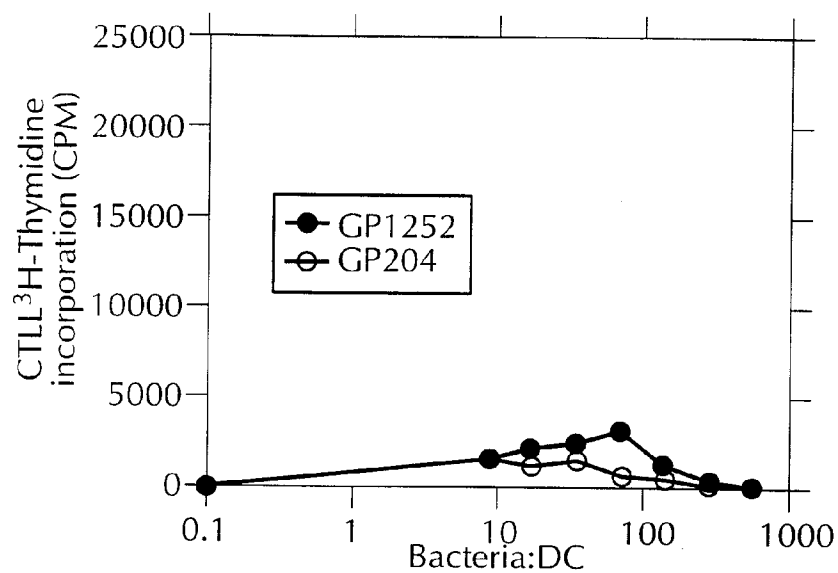
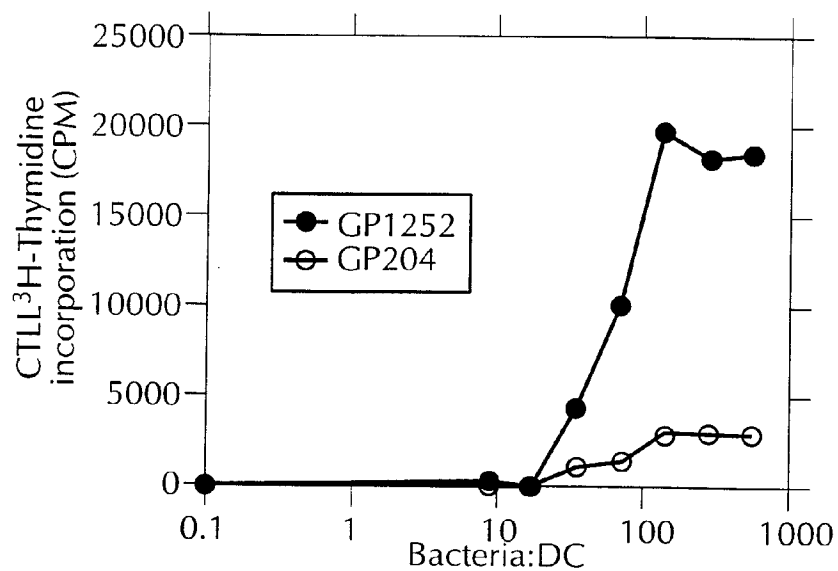


FIG. 2A



4/10

FIG. 3A**FIG. 3B**

5/10

FIG. 3C

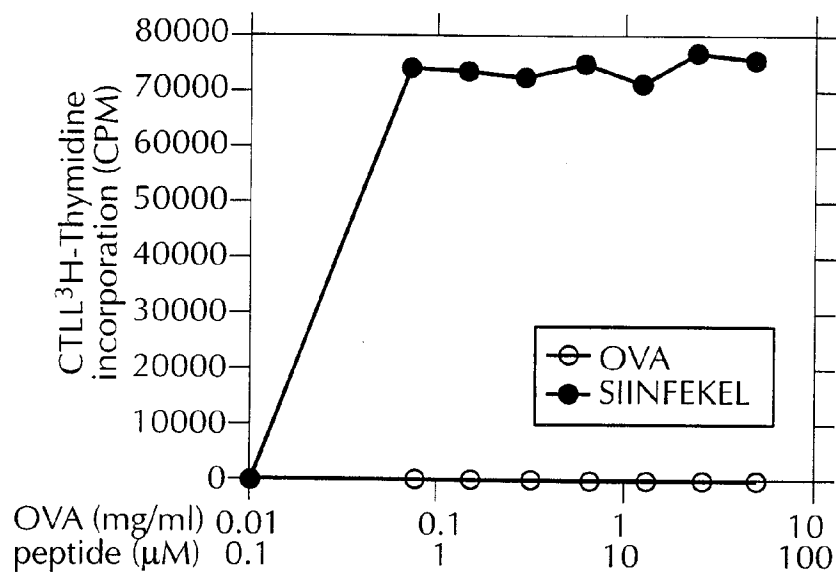
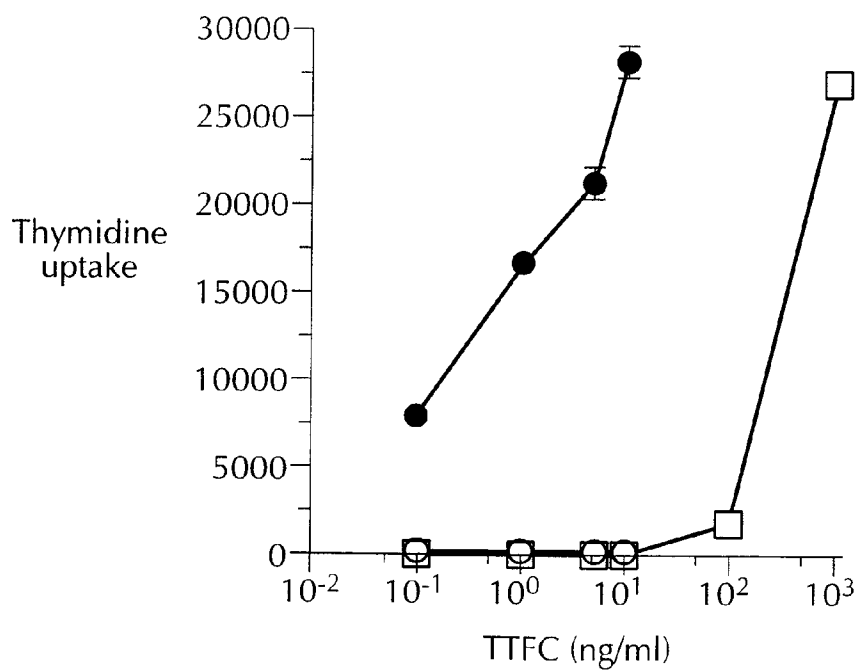
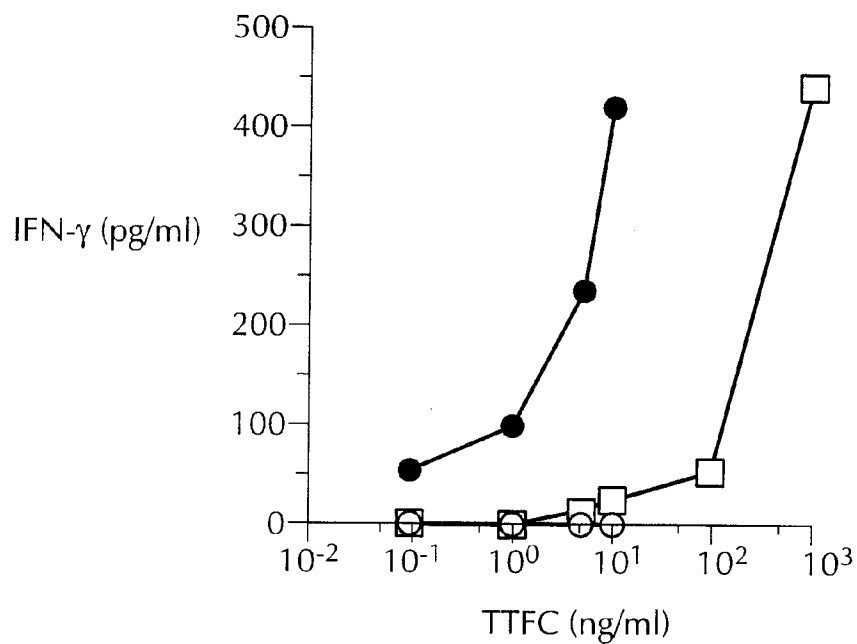
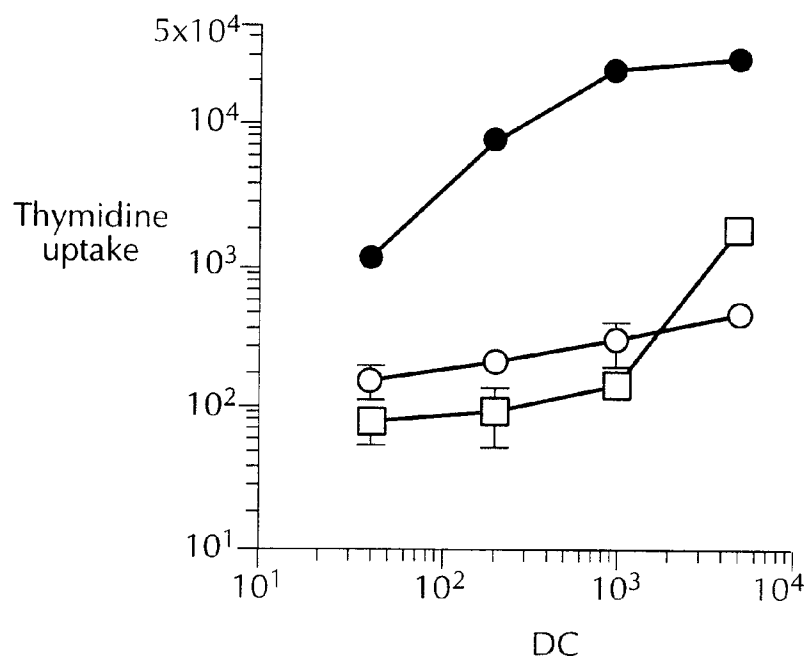


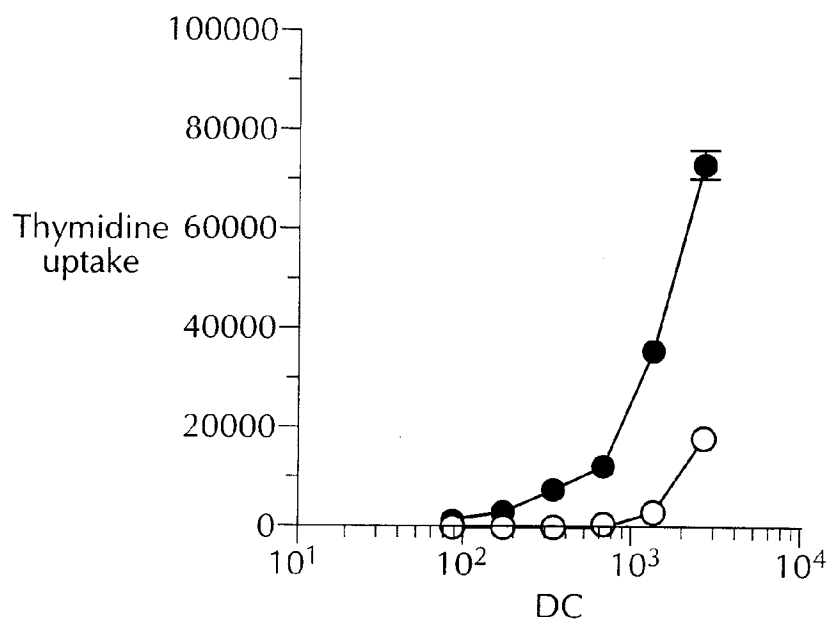
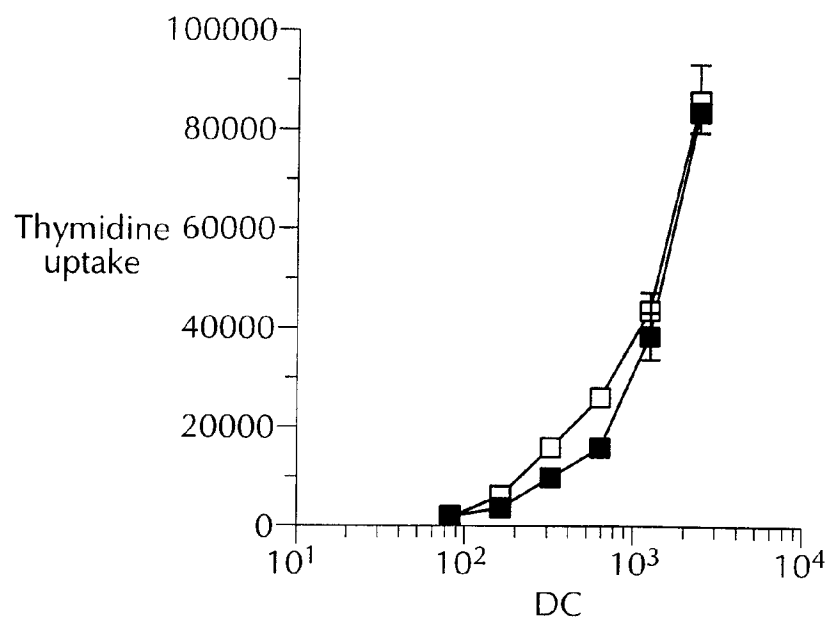
FIG. 4A



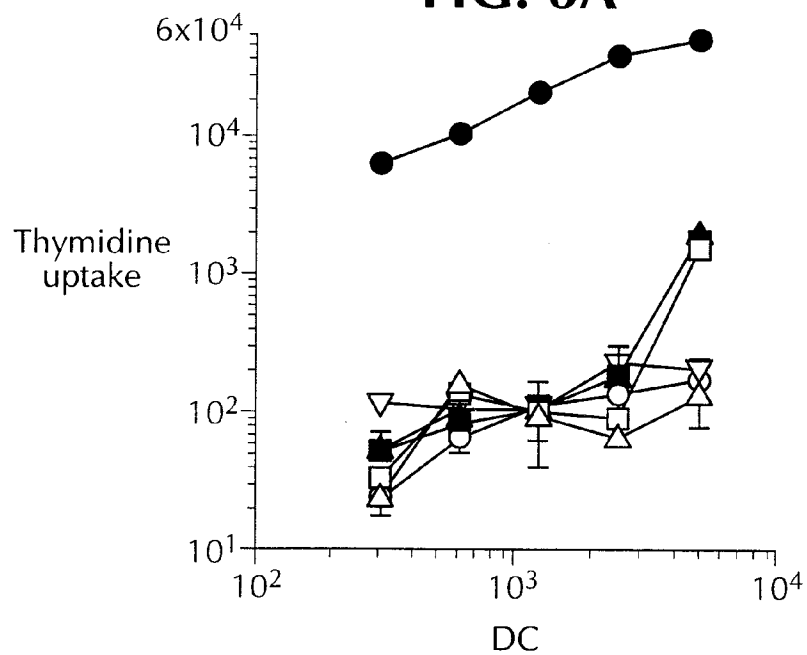
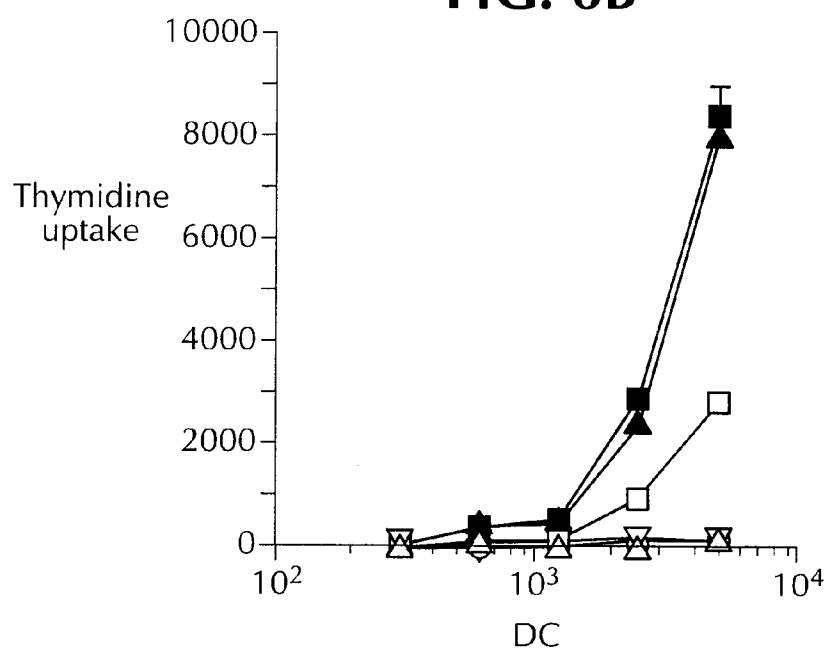
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FIG. 4B**FIG. 4C**

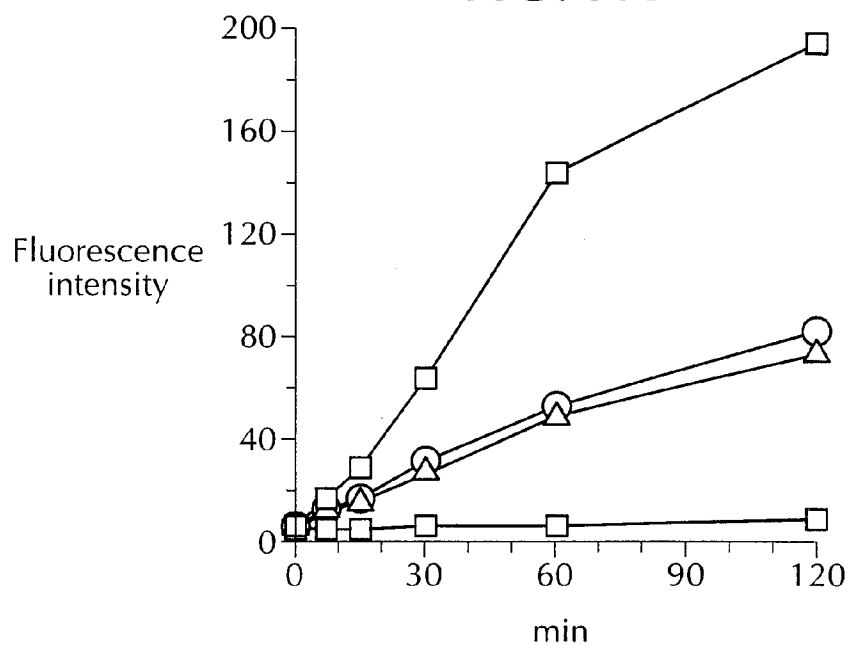
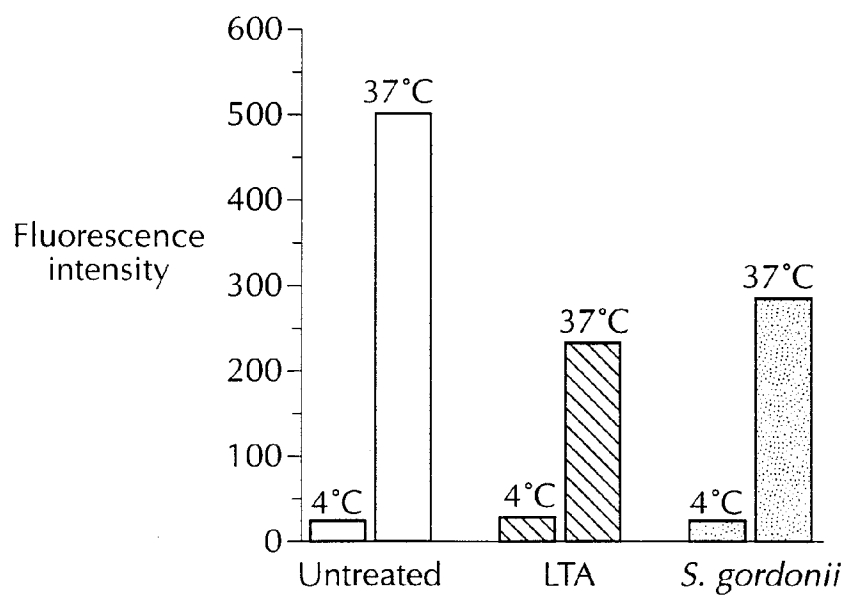
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FIG. 5A**FIG. 5B**

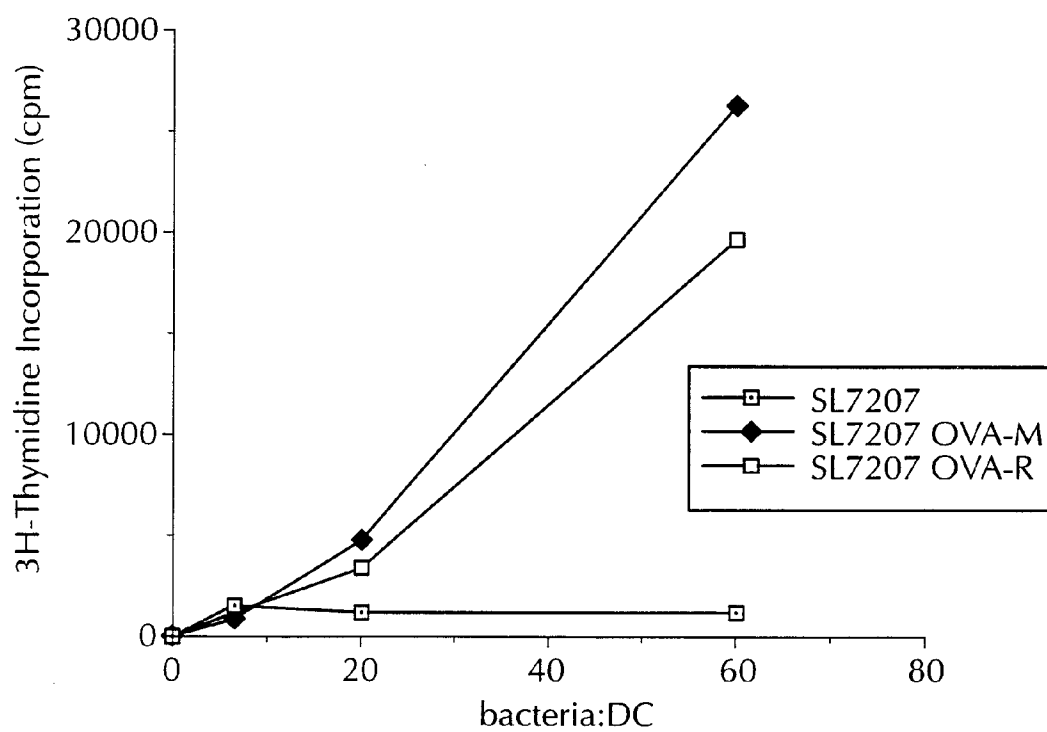
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FIG. 6A**FIG. 6B**

9/10

FIG. 7A**FIG. 7B**

10/10

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06627

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/06, 5/10

US CL : 435/325, 347, 355, 362, 372, 375, 377

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325, 347, 355, 362, 372, 375, 377

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG medicine index, APS, WEST:
terms: dendritic, cell, bacteria

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	SVENSSON, M. et al. Classical MHC class I peptide presentation of a bacterial fusion protein by bone marrow-derived dendritic cells. Eur. J. Immunol. January 1999, Vol. 29, No. 1, pages 180-188, see entire document.	1-5, 10-11, 16-18, 20-21, 23
X	SVENSSON, M. et al. Bone-marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. J. Immunol. 01 May 1997, Vol. 158, No. 9, pages 4229-4236, see entire document.	1-8, 10-18, 20-21, 23
X	SVENSSON, M. et al. Dendritic cells can process viable bacteria and present bacterial antigens on MHC-I molecules. Scand. J. Immunol. June 1996, Vol. 43, No. 6, page 723, abstract #121 in its entirety.	1-5, 7, 8, 10-19, 20-21, 23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 JUNE 1999

Date of mailing of the international search report

09 JUL 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06627

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DENIS, M. et al. Pleiotropic effects of the Bcg gene: I. Antigen presentation in genetically susceptible and resistant congenic mouse strains. J. Immunol. 01 April 1988, Vol. 140, No. 7, pages 2395-2400, see entire document.	1-8, 10-18, 20-21, 23